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The effect of cutaneous injury on a reproducible immersion challenge model for *Flavobacterium columnare* infection in channel catfish (*Ictalurus punctatus*)

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Abstract

We evaluated four methods of acute cutaneous injury: physical abrasion, hot and cold branding, and chemical scalds, to predispose channel catfish (*Ictalurus punctatus*) to columnaris disease, caused by the bacterium *Flavobacterium columnare*. Only physical abrasion (PA) and hot branding (HB) produced lasting alterations of epithelial architecture. Immersion challenge of PA or HB fish 10^8 cfu/ml virulent *F. columnare* at 29 ± 2 °C immediately after treatment resulted in death of most fish within the first 48 h. Immersion challenge 24 h after cutaneous injury resulted in 60% mortality with PA and 2% in HB. Cutaneous injury 48–72 h before challenge did not result in significant mortality. We quantified and modeled the median 96 h LD50 for PA and HB. The 96 h LD50 for abrasion ($10^{4.3 \pm 0.3}$ cfu/ml) was a log lower than for branded fish ($10^{5.0 \pm 0.4}$ cfu/ml). The LD50 for HB plus AS was $10^{5.8 \pm 0.3}$ cfu/ml, a log higher than for HB fish without Stresscoat™, an artificial slime agent (AS). Hot branding produced more consistent infection in terms of 96 h LD50s. Induction of experimental columnaris was dependent on the length of time between injury and pathogen exposure (<4 h), size of area disrupted on the epidermis, and dose of bacteria. A significant reduction in mortality and delay in death were noted when abraded fish were treated with AS and immediately challenged, suggesting that columnaris disease may be minimized or prevented by protecting a wounded catfish with AS, thus confirming the relationship of pathogenesis of *F. columnare* entering a wound.

Keywords: Columnaris disease; *Flavobacterium columnare*; Channel challenge model

1. Introduction

The development of an effective and reproducible bacterial immersion challenge model for *Flavobacterium columnare*, the causative agent of columnaris disease in commercially raised fish, in-

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cluding channel catfish (*Ictalurus punctatus*) is a crucial first step in the evaluation of new vaccines and therapheutics to prevent the disease. Immersion challenge models are well established for the black molly (*Poecila sphenops*), walleye, Atlantic salmon (*Salmon salar*), tilapia (*Tilapia* sp.) and eel (Morrison et al., 1981; Kuo et al., 1981; Hussain and Summerfelt, 1991; Decostere et al., 1999), but are still in development for channel catfish. There are at present three types of models: a standard immersion model with no stressor to predispose catfish to infection (Thomas-Jinu and Goodwin, 2004), a feed deprivation-based model (Klesius et al., 1999) and a cutaneous injury-based model (Bader et al., 2003). Cutaneous injury was used for that model because a relationship between handling abrasion and columnaris disease has been demonstrated for catfish (Hussain and Summerfelt, 1991; Hawke and Thune, 1992) and it had been historically used to mimic actual handling and netting injuries (Davies, 1922; Fish and Rucker, 1943). Investigators induced cutaneous injury in the host by abrading the skin with a sterile cloth immediately prior to challenge. The use of cutaneous injury significantly increased the likelihood of a successful challenge.

Typically models for virulence and toxicology are quantal tests designed to estimate the dose or concentration that affects 50% of the test organism at 96 h after challenge, i.e. lethal dose (LD50) or lethal concentration (LC50) at a given period of time (APHA, 1980). Lethal dose 50 models have been developed for other bacterial and viral fish pathogens for several fish species (Starliper et al., 1997; Fouz et al., 2002; Nusbaum et al., 2002), but none have yet been described for any immersion challenge models for columnaris disease in channel catfish. The quantification of the median 96-h LD50 for the injury-based (Bader et al., 2003) model is essential for the further development of that model.

As a logical extension of Bader et al. (2003) an additional series of studies were conducted (1) to determine which of four types of induced physical injury; physical abrasion, thermal branding, both hot and cold, and chemical abrasion, would result in injury that could predispose channel catfish to columnaris disease, and (2) to determine the median 96-h LD50 for the most efficacious cutaneous injury method(s). In a final objective we sought to use our standardized 96 h LD50 challenge model to study the effects of artificial mucus or slime (AS) on the susceptibility of channel to *F. columnare*.

2. Materials and methods

2.1. Fish

Channel catfish (*I. punctatus*), NWAC 103, were obtained from stocks held at the Auburn, Alabama USDA-ARS facility and were maintained in 57 L glass aquaria supplied with flow through water at 0.5 L/min and held at 29 ± 2 °C. The catfish had an average weight of 30 ± 5 g and average length of 15 ± 2 cm. Prior to use mucus and skin/muscle samples were randomly sampled from 20 catfish and cultured for *F. columnare* on modified Hsu-Shotts media (MHS) containing 2 mg/L tryptone, and no antibiotics (Bader et al., 2003) using standard methods (Bullock, 1994). A sample of each tissue was also processed for PCR detection of *F. columnare* using methods outline in Bader et al. (2003). Fish were determined to be culture and PCR negative for *F. columnare*. A dark and light period of 12:12 h was maintained and aeration was supplied through airstones. The fish were fed daily to satiation with catfish fingering chow (Cargill Inc., Guy, TX, USA).

2.2. Bacteria

A virulent and previously characterized isolate of *F. columnare* designated Agriculture Research Service strain 1 (ARS1) was used throughout this study (Bader et al., 2003). The bacteria were grown in MHS media with agitation (orbital shaker, 100 revolutions per minute) at 28 ± 1.0 °C for 24 h. Prior to challenge, 750 ml of MHS broth was centrifuged at $100 \times g$ for 30 min at 4 °C. The resulting bacterial pellets were adjusted to 1.0 optical density (OD) at 600 nm using a spectrophotometer (Model Smartspec 3000, Bio-Rad, Hercules, CA, USA). Bacteria at 1.0 OD were enumerated by triplicate spread plate counts on MHS plates using 0.1 ml of 10 fold serial dilutions of cell suspensions (Madigan et al., 2002). An OD of 1.0 was equivalent to 10^{10} colony forming units (cfu)/ml.

2.3. Experiments using physical injury without bacterial challenge

One hundred channel catfish were anesthetized with 120 mg/L tricane methylsulfanate (Argent Chemical Labs, Redmond, WA, USA) (MS 222) and divided into 5 groups of 20 fish each. Group 1 catfish were physically abraded by rubbing a sterile dry 4×4 cm piece of gauze cloth (4×4 Miasorb surgical sponges; Johnson and Johnson, Arlington, TX, USA) along the lateral line of the fish to create a 4×1.5 cm abraded area. Group 2 fish received a cold brand using a 10×0.1 cm long steel wire placed on dry ice for 20 s, then held on the side of the fish for 10 s. Group 3 fish were cauterized using a 10×0.1 cm long steel wire held over a Bunsen burner for 20 s then placed on the side of the fish for 10 s. Group 4 fish were treated with a sterile cotton tipped applicator (Puritan, Guilford, ME, USA) soaked in 0.1 M NH_4OH and drawn along a 10-cm line on the right side of the fish. Each of the cutaneously injured fish groups was placed in a clear tank after treatment. Group 5 fish were anesthetized, individually netted and moved to a clean tank, but were not physically injured and served as a control.

Samples for histopathology were taken from each group at 0 h, 24 h, 48 h and 72 h after treatment. Fish ($n = 5$/time point) were given lethal dose of MS 222 (300 mg/L) and 1 cm $\times$ 1 cm sections containing skin and underlying musculature were removed from the physically injured area on the side of the fish. Samples were preserved in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin and eosin.

The two methods of physical injury that produced the most extensive and reliable tissue damage (removal of the epidermis or damage to underlying musculature) were selected and used in *F. columnare* LD50 and AS challenge experiments to examine the effects of cutaneous injury on death loss due to columnaris disease.

2.4. Experiments using cutaneous injury with bacterial challenge

Separate experiments were conducted to evaluate the effects of physical abrasion and hot branding on fish mortality. Each experiment differed only in the cutaneous injury method used. Four hundred and eighty fish were anesthetized in 150 mg/L MS 222 and separated into eight cutaneous injury groups (60 fish/group): group 1 cutaneous injury (physical abrasion or hot branding) challenged after 0 h; group 2, cutaneous injury challenged after 4 h; group 3, cutaneous injury challenged after 24 h; group 4, cutaneous injury challenged after 48 h; group 5, cutaneous injury challenged after 72 h; group 6, cutaneous injury plus AS; group 7, no cutaneous injury; group 8, cutaneous injury no challenge. Fish from cutaneous injury groups 1–6 and 8 were physically abraded or hot branded, as previously described, while group 7 was kept uninjured and served as a cutaneous injury control. Group 6 was cutaneously injured and treated with AS, 7.5 ml AS/57 L water (Stresscoat™, Aquarium Pharmaceuticals, Inc., Chalfont, PA, USA) immediately after scarification. Group 8 remained unchallenged throughout the experiment and served as a challenge control. Immersion challenge involved draining each aquaria of fish to a volume of 10 L, adding 100 ml of 10^{10} cfu/ml (OD = 1.0), which is equivalent to 10^8 cfu/ml, holding the fish in the presence of the bacteria for 15 min., flushing the bacteria from the aquaria with flow-through water and finally returning each aquaria to the original volume of 57 L. The unchallenged group of fish was handled in the same manner as the other cutaneous injury groups, but was challenged only with MHS media. Fish mortality data of all the groups were recorded at 0, 18, 24, 48, 72, 96 h. These data were used to determine cumulative percent survival (CPS).

2.5. LD50 experiments

A lethal dose that kills 50% of challenged fish was determined for physically abraded and for branded cat fish in two separate experiments. Each experiment differed only in the physical injury method used on the fish. Eight hundred and forty fish were anesthesized in 150 mg/L MS 222 and divided into two groups; a cutaneously injured group (420 fish) and a non-cutaneously injured group (420 fish). The cutaneously injured group were physically abraded or hot branded and stock at a rate of 20 fish each into 21, 57 L glass aquaria (7 dose groups with three replicates each). The non-cutaneously injured group was stocked at a rate of 20 fish each into 21 (7 dose

groups with three replicates each), 57 L glass aquaria and served as a treatment control. All aquaria were challenged by immersion as previously described, with one of six 10-fold dilutions of *F. columnare* (10^8 to 10^3 cfu/ml) or sham challenged. Dilutions were made in MHS broth. The sham-challenged groups were only challenged with MHS broth only and served as a control group. Mortality following challenge was recorded daily for one week following challenge to determine CPS.

2.6. Artificial slime experiments

2.6.1. In vitro bacterial assay of an artificial slime agent

The artificial slime is reported to replace the natural protective slime layer of fish and is often used for the treatment of physical and environmental stress in aquarium fish at a volume of 5 ml/40 L water. To determine if AS had a bactericidal effect on *F. columnare* cells, bacteria were grown, as previously described to 10^{10} colony forming units (cfu/ml) or 1.0 OD in 125 ml MHS broth. The broth was divided into 6 tubes of 40 ml each. One set of three 40 ml tubes of the bacteria were then incubated with AS (5 μl/40 ml MHS) for 15 min, then 100 μl of each were plated in triplicate onto MHS agar plates and allowed to incubate at 28 °C for 48 h. Concurrently, the other set of three 40-ml tubes of the bacteria were then incubated with MHS only and incubated as previously described. These tubes served as controls.

2.6.2. Experiments using artificial slime agent and physical injury with bacterial challenge

Using data generated from the LD50 challenge experiments to determine the cutaneous injury method that had the highest LD50 (i. e. hot branding), an additional experiment was conducted to evaluate the disease mitigating effect of AS. Three hundred and seventy-eight fish were anesthetized in 150 mg/L MS 222 and divided into three cutaneous injury groups (126/group): group 1, hot branded; group 2, hot branded plus AS; group 3, non-injured. Group 1 was hot branded, as previously described. Group 2 was hot branded and treated with 7.5 ml/57 L AS immediately before immersion challenge. Group 3 did not receive was not hot branded or treated with AS and served as a control. Each cutaneous injury group was challenged by immersion, according to the previously described methods and immediately stocked into 27 aquaria (14 fish/aquaria) and subdivided into three treatment dose groups (42 fish/dose group). Each dose group was challenged one of three doses of *F. columnare*; 10^6, 10^5, and 0 cfu/ml. The 0 cfu ml/ml dose group, within each treatment group, was challenged only with sterile MHS media and served as control groups. Mortality following challenge was recorded for 96 h and was used to determine CPS.

2.7. Statistics

Cumulative percent survival was calculated by dividing number of surviving fish per time period by the total number of fish per aquaria and multiplying by 100. Cumulative percent survival was graphed on a Kaplan-Meir plot (PROC LIFETEST) versus time (SAS Institute, 1999). These data were then analyzed using analysis of variance (ANOVA) with general linear model (PROC GLM) and was tested for significance using Duncan's multiple range test (SAS Institute, 1999). Probit analysis was used to determine 96 h LD50s and significant differences between treatments over time (SAS Institute, 1999). A paired *t*-test was used to test for significance in the in vitro bactericidal assay of an artificial slime agent. Statistical significance is declared at $P \leq 0.05$ (Steel et al., 1997).

3. Results

In the experiments to compare effects of each cutaneous injury method, catfish with cutaneous injuries caused by cold branding and chemical abrasion did not differ from control fish (Fig. 1A) and showed little or no damage to the epidermis or underlying musculature in the 72-h study (data not shown). In contrast, tissue samples from catfish that were physically abraded and hot branded (Fig. 1B and D, respectively) showed extensive tissue damage immediately following injury (0 h). The dermis was intact, but epidermis was partially destroyed by the treatment. At 24 h following injury the physical abraded catfish (Fig. 1C) showed injuries similar to 0 h, while the hot branded fish showed a reorganized epithelium with a swollen dermis (Fig. 1E). At 72

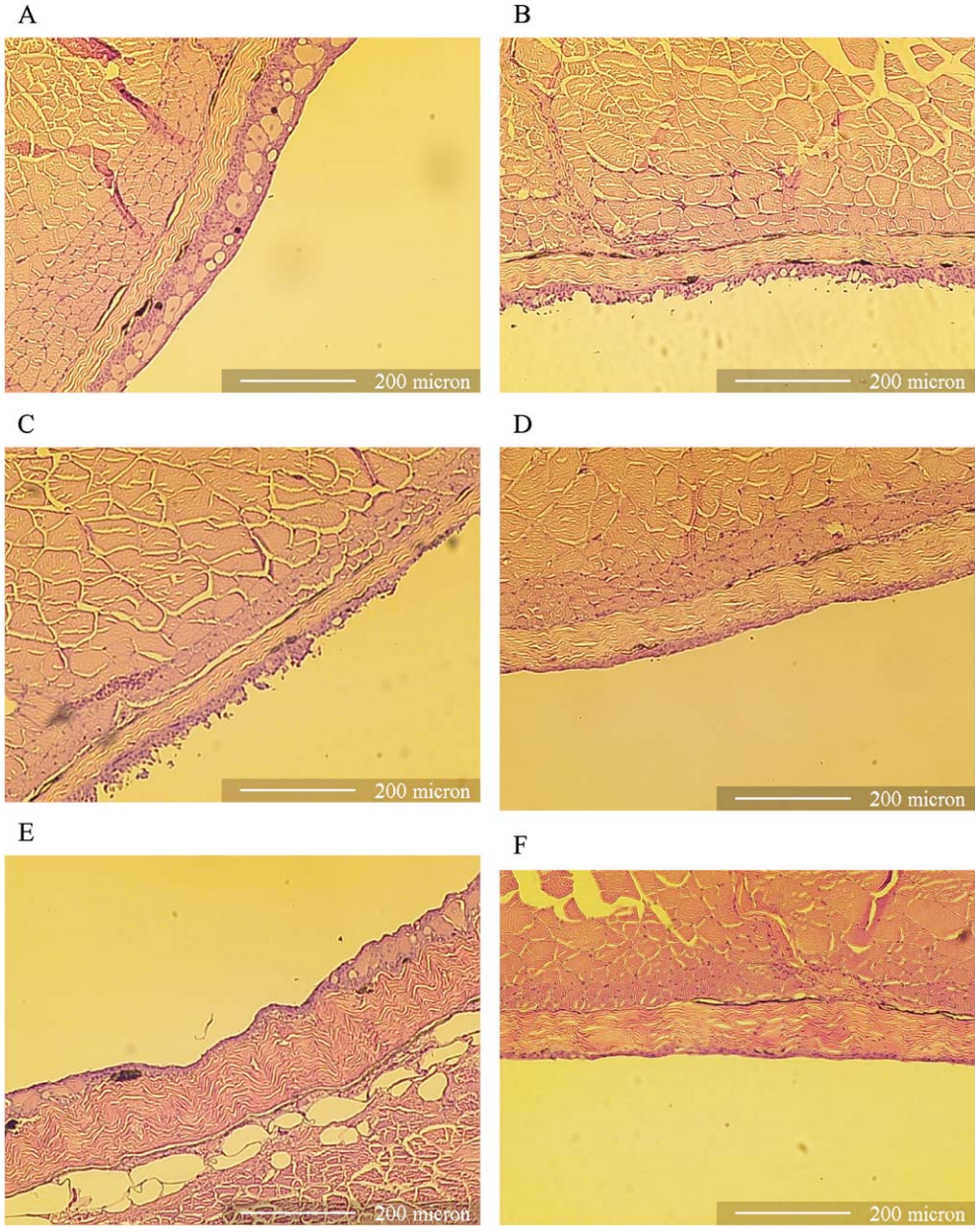

Fig. 1. Hematoxylin and eosin stained histological sections of channel catfish tissue from site of physical cutaneous injury. (A) Skin of control (non-abraded) channel catfish. (B) Skin of channel catfish 0 h after physical abrasion with a sterile dry 4 × 4 cm piece of gauze cloth. The dermis is intact but the epidermis was partially destroyed by the abrasion. (C) Skin of channel catfish 24 h after physical abrasion. (D) Skin of channel catfish 0 h after hot branding using a heated 10 × 0.1 cm long steel wire. The dermis is compressed and the epidermis is swollen. (E) Skin of channel catfish 24 h after being heat branded. (F) Skin of channel catfish 72 h after physical abrasion.

h following injury the physical abraded catfish showed almost total tissue reorganization (Fig. 1F). Based on the initial histopathology study, only two cutaneous injury methods, hot brand and physical abrasion, were evaluated in conjuction with bacterial challenge with *F. columnare*.

A Kaplan-Meir plot of cumulative percent survival (represented as a column chart) for fish physically abraded at 0, 4, 24, 48, or 72 h before challenge is shown in Fig. 2 for 0, 18, 24, 48, 72, and 96 h following challenge (10^8/ml). All fish that were challenged 0 h after physical abrasion died in less than 18 h, while all fish that were physically abraded 4 h before challenge died in 48 h. Fish that were physically abraded 24, 48 and 72 h before challenged survive through the 96 h. The artificial slime agent increased the CPS over time and delayed onset of death. All non-injured fish died within the first 48 h of challenge. No mortality was recorded for non-challenged fish in any cutaneous injury group.

A Kaplan-Meir plot of cumulative percent survival (represented as a column chart) for fish physically abraded at 0, 4, 24, 48, or 72 h before challenge is shown in Fig. 3 for 0, 18, 24, 48, 72, and 96 h following challenge (10^8/ml). Fish that were challenged 0 h and 4 h after hot branding died in less than 48 h. All fish that were hot branded 24 h before challenge died before 72 h, while those fish that were hot branded 48 h before challenge survived through the end of the experiment (96 h). The artificial slime agent increased the CPS over time and delayed onset of mortality. All non-injured fish died within the first 48 h of challenge. No mortality was recorded for non-challenged fish in any cutaneous injury group.

Ninety-six hour LD50 data for the physically abraded, hot branded and uninjured control fish is summarized in Table 1 and shows significantly different LD50s. The LD50 for hot branded fish was a log higher than for physically abraded fish. Based on this data, hot branding was chosen for use in the AS experiments. No LD50s data could be calculated for the uninjured control fish group because all fish died before 96 h. No mortality was recorded for the sham-challenged controls. The results of the in vitro bac-

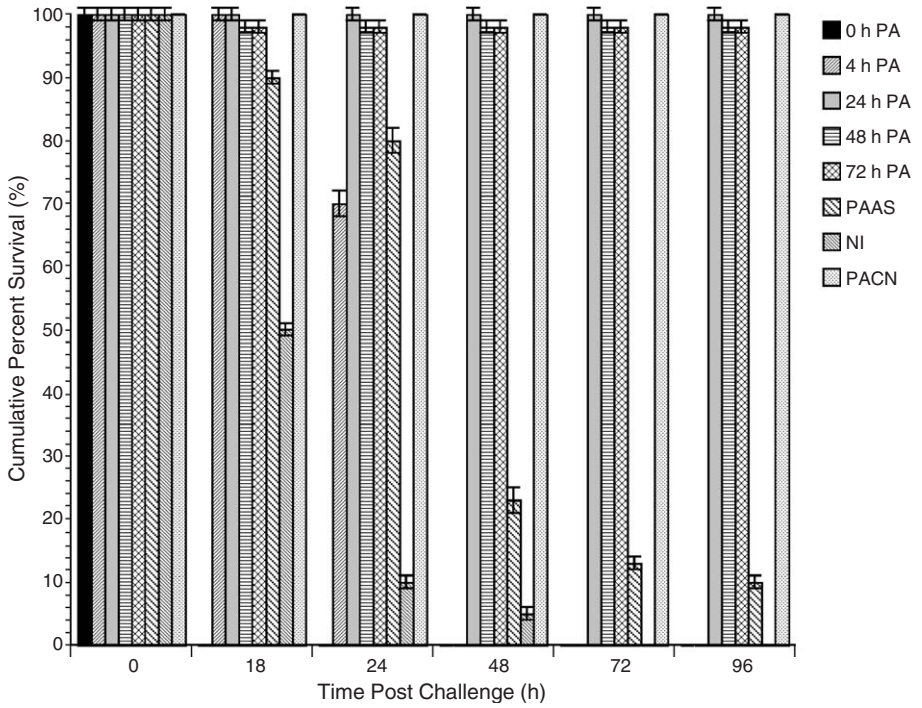

Fig. 2. Column chart of cumulative percent survival for 8 groups of channel catfish ($N=3$). Groups 0 h physically abraded (PA) through 72 h PA were physically abraded with a sterile dry 4 × 4 cm piece of gauze cloth and challenged at 0, 4, 24, 48, and 72 h following injury. The physically abraded and artificial slime agent (PAAS) treated group were abraded, treated with 7.5 ml/57 L of a commercial artificial slime agent and then challenged with 10^8 CFU/ml *Flavobacterium columnare*. An uninjured group (NI) of catfish was not injured and was challenged. A physically abraded group of fish was sham challenged with Modified Hsu Shotts media only and served as a control (PACN). Standard errors of the mean are indicated by bars on the graph.

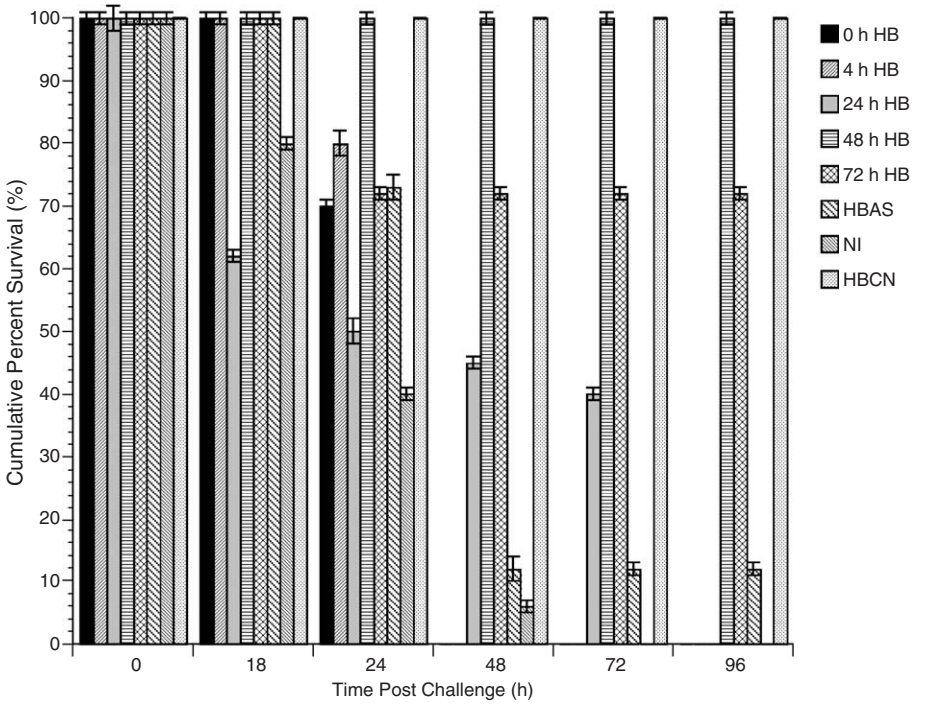

Fig. 3. Column chart of cumulative percent survival for 8 groups of channel catfish ($N=3$). Groups 0 h hot branded (HB) through 72 h HB were branded with a heated 10×0.1 cm long steel wire and challenged at 0, 4, 24, 48, and 72 h following injury. The hot branded and artificial slime agent (HBAS) treated group were abraded, treated with 7.5 ml/57 L of a commercial artificial slime agent and then challenged. Groups were challenged with 10^8 CFU/ml *Flavobacterium columnare*. An uninjured group (NI) of catfish was not injured and was challenged. A hot branded group of fish was sham challenged with modified Hsu Shotts media only and served as a control (HBCN). Standard errors of the mean are indicated by bars on the graph.

teriocidal assay of AS agent on *F. columnare* are summarized in Table 2 and shows significant bactericidal activity. The effect of AS on *F. columnare* infection was evaluated using the established immersion challenge LD50 model using cutaneous injury (hot branding) (Table 3) and found to increase the 96 h LD50s significantly. No mortality was recorded for the 0 cfu ml/ml dose group.

Table 1
96 h LD50s for a *Flavobacterium columnare* immersion challenge experiment with channel catfish which were first physically abraded with a sterile dry 4×4 cm piece of gauze cloth or hot branded with a heated 10×0.1 cm long steel wire and then challenged with 10^8 CFU/ml *F. columnare*

Treatment	Log dose	C.I.	Number fish tested
Physical abrasion	4.3 a	4.0–4.5	60
Hot brand	5.0 b	4.6–5.4	60
Control	ND	ND	60

Data represents 96 h LD50s with 95% confidence intervals at (C.I). Different letters denote significance. No LD 50 data (ND) was obtained from the uninjured control group challenged with 10^8 CFU/ml *F. columnare*, because 100% of catfish died before 96 h.

4. Discussion

Our study demonstrates that various types of cutaneous injury produce different degrees epithelial damage and the area of that damage is the major factor predisposing catfish to columnaris infection during immersion challenge. Histological evaluation of tissue sections from the sites of four types of artificially

Table 2
Number of *Flavobacterium columnare* colonies following incubation with an artificial slime (AS) agent 15 min at 28 °C

AS (SE)	Control (SE)
$3.2\times10^6(\pm.5)^a$	$4.6\times10^8(\pm.2)^b$

Different denote significance from control group at $p\leq0.05$. $N=3$.

Table 3
96 h LD50s for a *Flavobacterium columnare* immersion challenge experiment with channel catfish which were hot branded with a heated 10 × 0.1 cm long steel wire treated with 7.5/57 ml of an artificial slime (AS) agent and then challenged with three doses of *F. columnare*; 10^6, 10^5, 0 cfu/ml

Treatment	Log dose	C.I.	Number fish tested
Hot brand + Without AS	4.8 a	4.6–5.1	42
Hot brand + AS	5.83 b	5.6–6.1	42
Control	3.5	3.3–3.8	42

Control fish were challenged with 0 CFU/ml *F. columnare*. Data represents 96 h LD50s with 95% confidence intervals at (C.I.). Different letters denote significance from uninjured control.

induced cutaneous injury, physical abrasion, thermal branding, both hot and cold, and chemical abrasion, in absence of the pathogen differed from each other in discernable damage, particularly to the underlying musculature. This damage was most evident in hot branded and physically abraded catfish particularly in the hours following injury and by 72 h was nearly healed. In actual bacterial challenge studies, hot branded and physically abraded catfish were also observed to be most susceptible to infection. Both of these methods created large areas of cutaneous damage to the epithelial layer of the skin and we believe it is this that predisposes the catfish to infection. Fish that were allowed as little as 4 h for regeneration of the epithelium, independent of the condition of the underlying musculature, had mortality rates decrease from 100% to 30%. No mortality occurred in fish that were allowed three days between physical injury and challenge, regardless of the method of physical injury. This is consistent with previous studies that showed that physical abrasion results in higher rates of infection (Kuo et al., 1981; Morrison et al., 1981; Hussain and Summerfelt, 1991; Bader et al., 2003). It meets the criteria, established previously by Bader et al. (2003), for establishing a balance between mimicking natural forces and producing clinically sick animals. The interruption of the protective epithelial layer appears to create a portal of entry for *F. columnare*, and loss of the mucus or slime layer present on the epidermis, which normally serves as a barrier to bacterial infection (Ellis, 2001), may prime the fish for attachment of the bacteria.

A number of studies have shown the usefulness 96 h median dose, LD50 models to evaluate treatments effects in channel catfish (Jantrarotai and Lovell, 1990; Hanson and Grizzle, 1985; Thune et al., 1982). We have shown that such a channel catfish model, using cutaneous injury to predispose the host to infection, works well and can be standardized. Our studies also provide evidence as to how cutaneous injury, physical abraded and hot branded may influence columnaris infection. When we modify the number of bacteria entering the host by altering the denuded area, we find that the LD50 found in physically abraded fish was roughly one log lower than that found with branding because the larger area of disruption of the epidermis permitted more bacteria to enter the wound. In practical terms, both physical abrasion and hot branding produce the desired effect on the host of predisposing it to *F. columnare* infection and our results support the utility of both. There are advantages and disadvantages with each method and the choice of method will depend on the desired outcome of the researcher. Hot branding can result in a more repeatable sized lesion, because the researcher can manipulate the lesion size by changing the size of the branding instrument. However, this method can bring with its use animal care and ethical concerns and may not achieve the balance between mimicking natural forces and producing clinically sick animals. The physical abrasion method, while achieving a better balance between mimicking natural forces and producing clinically sick animals, is also not perfect, because it can sometimes cause inconsistent lesions and death rates. Inconsistent lesions and death rates result from the fact that the production of consistent lesions by the use of a dry sterile gauze cloth is difficult and can differ from person to person, as hand size and pressure varies with the individual. While the choice of cutaneous injury method will always be up to the researcher, the inclusion of such a method is a most for a standardized 96 h LD50s immersion challenge disease in channel catfish.

The 96 h LD50 data for AS is not only useful in demonstrating how to perform 96 h LD50s immersion challenge experiments using hot branding to test therapeutics, but also sheds some light onto the columnaris disease process in catfish, generally. Our artificial slime data shows that the AS is bactericidal to *F. columnare* and that the replacement of the catfish's natural slime layer with an artificial slime product can partially protect catfish from

high concentrations of *F. columnare* by decreasing the number of entering bacteria. When only a 2-cm brand was used, fish challenged with a slightly lower concentration of bacteria and treated with AS showed dramatic improvements in survival time over fish under identical conditions which were not treated with AS, thus strengthening the dose-related-response hypothesis. The determining factor as to whether or not replacement of the slime layer is effective is probably related to the total area of compromised tissue on the surface of the fish. Because breaks in the epithelial layer incurred during handling are generally small, replacement of the slime layer may prove to be an effective means of preventing columnaris disease as a result of handling injury in channel catfish.

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